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Novel sources of β-glucanase for the enzymatic degradation of schizophyllan☆

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ABSTRACT

Schizophyllan is a homoglucan produced by the fungus *Schizophyllum commune*, with a β-1,3-linked backbone and β-1,6-linked side chains of single glucose units at every other residue. Schizophyllan is commercially produced for pharmaceutical and cosmetics uses. However, surprisingly little information is available on the biodegradation of schizophyllan. Enzymes that attack schizophyllan could be useful for controlled modifications of the polymer for novel applications. Enrichment cultures were used to isolate 20 novel fungal strains from soil samples, capable of growing on schizophyllan as a sole carbon source. Three additional strains were isolated as contaminants of stored schizophyllan solutions. Strains showing the highest levels of β-glucanase activity were identified as *Penicillium simplicissimum*, *Penicillium crustosum*, and *Hypocrea nigricans*. β-glucanases also showed activity against the similar β-glucans, laminarin and curdlan. By comparison, commercial β-glucanase from *Trichoderma longibrachiatum* and laminarinase from *Trichoderma* sp. showed lower specific activities toward schizophyllan than most of the novel isolates. β-glucanases from *P. simplicissimum* and *H. nigricans* exhibited temperature optima of 60 °C and 50 °C against schizophyllan, respectively, with broad pH optima around pH 5.0. Partial purifications of β-glucanase from *P. simplicissimum* and *P. crustosum* demonstrated the presence of multiple active endoglucanase species, including a 20–25 kD enzyme from *P. simplicissimum*.

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1. Introduction

Schizophyllan is a polysaccharide produced by *Schizophyllum commune*, a white-rot fungus and ubiquitous mushroom. It is a homoglucan with a β-1,3-linked backbone and single β-1,6-linked glucose side chains at every other residue [1,2]. Schizophyllan acts as a biological response modifier and a non-specific stimulator of the immune system. It is used in vaccines, anti-cancer therapies, and as a bioactive cosmetics ingredient. Schizophyllan can form oxygen-impermeable films for food preservation [3]. It also has been tested for use in enhanced petroleum recovery [2,4].

As a natural polysaccharide, it can be assumed that schizophyllan is biodegradable, and many of its applications rely on this assumption. However, surprisingly little information is available on the biodegradation of schizophyllan. *S. commune* has been reported to produce endo-β-1,3-glucanase [5], and Rau [2] proposed that the organism can consume schizophyllan as a carbon source, contributing to a loss of polysaccharide molecular weight in late cultures. Lo et al. [6] described β-glucosidases from *S. commune*. Fontaine et al. [7] reported that schizophyllan was slightly hydrolyzed by one of two exo-β-1,3-glucanases associated with the cell walls of *Aspergillus fumigatus*. On the other hand, Kanzawa et al. [8] found that exo-β-1,3-glucanase from *Bacillus circulans* rapidly hydrolyzed curdlan and laminarin, but did not attack schizophyllan. Tanji et al. [9] reported that schizophyllan was partially degraded at a very slow rate in rats, to lower molecular weight forms of <10,000 that were excreted in urine. It is potentially valuable to identify enzymes that attack schizophyllan, particularly for use in controlled modifications of the polymer for novel applications.

In the current study, 23 novel strains were isolated that were capable of growing on schizophyllan as a sole carbon source. Strains showing the highest activities against schizophyllan were identified, and β-glucanase activities were characterized. Results indicate that novel fungal isolates are promising sources of schizophyllan-degrading enzymes.

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Table 1
β-glucanase activity against schizophyllan produced by newly isolated strains[a]

Strain number	Isolation sites (near Peoria, Illinois)	β-glucanase (U activity/mL)[b]
1-1	Woodland	0.24 ± 0.02
1-2		0.16 ± 0.03
1-3		0.17 ± 0.03
2-1	Prairie grassland	0.005 ± <0.001
2-2		<0.001
2-3		0.003 ± <0.001
2-4		0.001 ± <0.001
3-1	Hayfield/woodland	<0.001
3-2	border	<0.001
4-1	Pond shore	0.007 ± <0.001
4-2		0.001 ± <0.001
6-1	Clay soil	0.008 ± <0.001
6-2		0.002 ± <0.001
6-3		0.21 ± 0.05
6-4		0.19 ± 0.01
7-1	Marshland	<0.001
7-2		0.08 ± 0.02
8-1	Backyard/gardening	0.007 ± <0.001
8-2	area	0.003 ± <0.001
8-3		0.003 ± <0.001
9-1	Laboratory contaminant	0.026 ± <0.001
9-2		0.032 ± <0.001
9-3		0.029 ± <0.001

[a] Strains were isolated from enrichment cultures containing schizophyllan as a sole carbon source.
[b] Pure cultures were grown for 7 days in basal medium containing 1.0% schizophyllan as a sole carbon source.

2. Methods

2.1. Isolation of novel schizophyllan-degrading strains

Soil samples from the area of Peoria, Illinois were serially diluted according to Leathers et al. [10]. Specifically, 2.0 g of soil was diluted into 198 mL of sterile 0.2% agar in distilled water (water agar). This was shaken vigorously, and then 10 mL was transferred to 90 mL of water agar and mixed well. One milliliter of this suspension was then transferred into 9 mL of water containing 0.01% Tritron X-100. Aliquots (0.1 mL) of these final dilutions were used to inoculate 10 mL enrichment cultures containing 1.0% (w/v) schizophyllan (cosmetic grade, European Technologies, Inc., Denver, CO) as a sole carbon source in basal medium composed of 0.67% (w/v) yeast nitrogen base, 0.5% (w/v) KH_2PO_4, and 0.2% (w/v) bacto-asparagine (Difco Laboratories, Detroit). Enrichment cultures were grown for 7 days at 28 °C and 200 rpm. Serial dilutions were made onto solid medium containing potato dextrose agar (PDA, Difco), and isolates were single-colony purified at least three times. In addition, three strains were isolated as laboratory contaminants of stored schizophyllan solutions (Table 1).

2.2. Sequence identification of strains

Conidia were floated free of the mycelium with 2–3 mL of 70% ethanol and concentrated by brief centrifugation in a micro-centrifuge. Conidial pellets were suspended in 400 μL of CTAB buffer [11] in a 1.5 mL microfuge tube containing about 400 mg of 0.5 mm diameter glass beads. Cell walls were broken by vortex mixing of the glass beads and conidia. Chloroform (0.4 mL) was added to extract proteins and the aqueous and organic phases were separated by centrifugation. The aqueous phase was transferred to a clean microfuge tube, precipitated by addition of an equal volume of isopropanol and collected by centrifugation. The resulting pellet was rehydrated in 100 μL buffer (1 mM Tris, 0.1 mM EDTA, pH 8.0). The DNA preparation was diluted 10 to 100 times in sterile distilled water for use in PCR amplifications. Beta tubulin was amplified using the primers and procedures of Glass and Donaldson [12]. Alternatively, DNA isolation was carried out using a ZR Fungal/Baterial DNA kit following the procedure according to Nawrot et al. [13], and strains were identified by the sequence of their 28s rRNA genes. Primers were NL-1 (59-GCATATCAATAAGCGGAGGAAAAG) and NL-4 (59-GGTCCGTGTTTCAAGACGG) as described by O'Donnell [11].

2.3. Culture conditions for β-glucanase production on schizophyllan

Strains were grown on PDA slants at 28 °C for 7–10 days. An approximately 7 mm × 7 mm square of mycelium was used to inoculate 10 mL of CB basal medium containing 1.0% (w/v) commercial schizophyllan in a 50 mL flask with three 10 mm glass beads. Cultures were incubated at 200 rpm for 7 days at 28 °C, then centrifuged at 3220 × *g* to produce cell-free culture supernatants. Mycelial pellets were dried at 60 °C for 48 h. All experiments were carried out in triplicate and standard deviations are shown.

2.4. Enzyme and protein assays

Quantitative β-glucanase assays were performed by the dinitrosalicylic acid (DNS) method [14] as modified by Leathers et al. [15]. Samples (5–20 μL) were incubated in a total volume of 205 μL containing 0.5% (w/v) substrate (schizophyllan or another β-glucan) in 50 mM sodium acetate buffer, pH 5.0, at 28 °C. Sample dilutions and incubation times were adjusted to ensure results were within the linear range of the assays. One unit of enzyme activity is defined as the amount of enzyme necessary to release 1 μmole of glucose equivalents per min under the conditions tested. Schizophyllan (cosmetic grade) was purchased from European Technologies, Inc., Denver, CO. Other β-glucan substrates (laminarin from *Laminaria digitata*, paramylon from *Euglena gracilis*, curdlan from *Agrobacterium* sp. (*Alcaligenes faecalis*), and barley β-glucan) were from Sigma-Aldrich, St. Louis. Commercial cellulases from *Aspergillus niger* and *T. viride* (Cellulysin) were from Calbiochem (La Jolla, CA). Commercial β-glucanases from *A. niger*, *T. longibrachiatum*, and *Bacillus subtilis*, as well as laminarinase from *Trichoderma* sp., were from Sigma–Aldrich. Temperature and pH optima were performed using the same assay. For studies of pH optima, substrate buffer was titrated to the desired test pH with acetic acid or sodium hydroxide before digestion, then returned to pH 5 before assays were developed, since the DNS assay is pH sensitive. Enzyme values are the mean of triplicate cultures and are characteristic of repeated experiments.

Rapid, semi-quantitative β-glucanase assays were performed using a solid medium plate assay. Samples (4 μL) were spotted directly onto the surface of freshly prepared plates containing 0.7% (w/v) Phytagel (Sigma–Aldrich Co., St. Louis) and 0.05% (w/v) commercial schizophyllan in 50 mM sodium acetate buffer, pH 5.0. Plates were incubated overnight and stained with an aqueous solution of 1 mg Congo red/mL for 45 min, then destained with 1.0 M NaCl for 30 min. Enzyme activity was observed as a clear zone. Laminarinase from *Trichoderma* sp. was used as a positive control.

Extracellular protein was estimated by the Bradford method [16], with bovine serum albumin as the standard.

2.5. Enzyme purification by fast protein liquid chromatography (FPLC)

Enzyme samples were purified using a fast protein liquid chromatography system (Biologic Duoflow, Bio-Rad, Hercules, CA). Cell-free culture supernatants were applied to a 5.0 mL High Q anion exchange column (Bio-Rad) equilibrated in 50 mM sodium acetate buffer, pH 5.0. Bound protein was eluted with an increasing gradient of 0.0 to 1.0 M NaCl in the same buffer. Alternatively, samples were adjusted to 1.0 M $(NH_4)_2SO_4$ by direct addition of solid $(NH_4)_2SO_4$ and then applied to a Phenyl Sepharose column (1.5 × 10 cm, GE Healthcare, Piscataway, NJ) equilibrated with 1.0 M $(NH_4)_2SO_4$ in 50 mM sodium acetate buffer, pH 5.0. Bound protein was eluted with a decreasing gradient from 1.0 M to 0.0 M $(NH_4)_2SO_4$ in the same buffer.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Active enzyme fractions from liquid chromatography were desalted using protein desalting spin columns (Pierce, Rockford, IL). Samples were denatured by heating for 4 min at 95 °C in 2× SDS sample buffer (4.0% (w/v) SDS, 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue, 25% (w/v) 0.5 M Tris–HCl pH 6.8 and 5.0% (v/v) β-mercaptoethanol) and applied to an SDS-PAGE gel (3.0% stacking, 10% resolving). After electrophoresis at 100 V for approximately 1 h, the SDS PAGE gel was stained with SYPRO Ruby protein gel stain (Invitrogen, Grand Island, NY) for 16 h and rinsed with deionized water. Stained gels were visualized by UV transillumination.

2.7. Zymogram analysis

Samples from cell-free culture supernatants and liquid chromatography fractions were denatured as described above and applied to a 10% SDS-PAGE gel containing 0.04% (w/v) commercial schizophyllan within the gel matrix. Following electrophoresis, the gel was incubated in 50 mM sodium acetate buffer, pH 5.0 at 28 °C for 20 h, stained with an aqueous solution of 1.0 mg Congo Red/mL for 30 min, and destained with 1.0 M aqueous NaCl for 30 min. After rinsing with deionized water, enzyme activity was visualized on the gel as clear bands against a red background of stained schizophyllan. The clear band resulted from the degradation of schizophyllan.

Table 2
Identification of novel strains producing β-glucanase activity against schizophyllan.

Isolate number	Strain number	Species designation	Match[a]	Colonial morphology
1-1	NRRL 62550	*Penicillium simplicissimum*	566/569	Low velutinous, conidia celadine green becoming olive centrally, abundant clear exudates, reverse cream
1-2	NRRL 62551	*Hypocrea nigricans*	565/565	Rapidly spreading dark blue-green, numerous scattered cleistothecia
1-3	NRRL 62552	*H. nigricans*	558/558	Rapidly spreading dark blue-green, numerous scattered cleistothecia
6-1	NRRL 62553	*Aspergillus flavus*	566/566	Velutinous pea-green, clear exudates, numerous sclerotia, reverse creamy
6-2	NRRL 62554	*A. alliaceus*	566/566	Floccose, clear exudates, central stromata
6-3	NRRL 62555	*H. nigricans*	558/558	Rapidly spreading dark blue-green, numerous scattered cleistothecia
6-4	NRRL 62556	*H. nigricans*	560/560	Rapidly spreading dark blue-green, numerous scattered cleistothecia
9-1	NRRL 62557	*Penicillium crustosum*	564/564	Low spreading velutinous, artemesia green, reverse pale yellow-orange
9-2	NRRL 62558	*P. crustosum*	564/564	Low spreading velutinous, artemesia green, reverse pale yellow-orange
9-3	NRRL 62559	*P. crustosum*	578/579	Low spreading velutinous, artemesia green, reverse pale yellow-orange

[a] Sequence match to proposed species designations in GenBank database.

3. Results and discussion

3.1. Isolation of novel organisms capable of growth on schizophyllan as a sole carbon source

Since schizophyllan is produced by the ubiquitous mushroom, *S. commune*, novel organisms capable of degrading schizophyllan were sought in soil samples from 7 diverse environments near Peoria, IL (Table 1). Soil dilutions were used to inoculate enrichment cultures containing commercial schizophyllan as a sole carbon source. All soil samples tested provided good growth on this medium, and 20 isolates were purified from these cultures by repeated single-colony isolations (Table 1). In addition, three organisms were isolated as laboratory contaminants of stored schizophyllan solutions.

Purified strains were cultured in basal medium containing schizophyllan as a sole carbon source. All cultures exhibited good growth on schizophyllan. Mycelial dry weights ranged from 25–100 mg from cultures initially containing 100 mg schizophyllan (data not shown). Cell-free culture supernatants were assayed for β-glucanase activity against commercial schizophyllan (Table 1). Five of 20 soil isolates exhibited β-glucanase activity of approximately 0.2 U/mL (Table 1), while the three laboratory contaminants showed activities of about 0.03 U/mL. The remainder of the isolates showed lower β-glucanase activities, some below the limits of detection by this assay (<0.001 U/mL). The 8 best producers of β-glucanase activities against schizophyllan were chosen for further study. Low producer isolates 6–1 and 6–2 were included as negative controls.

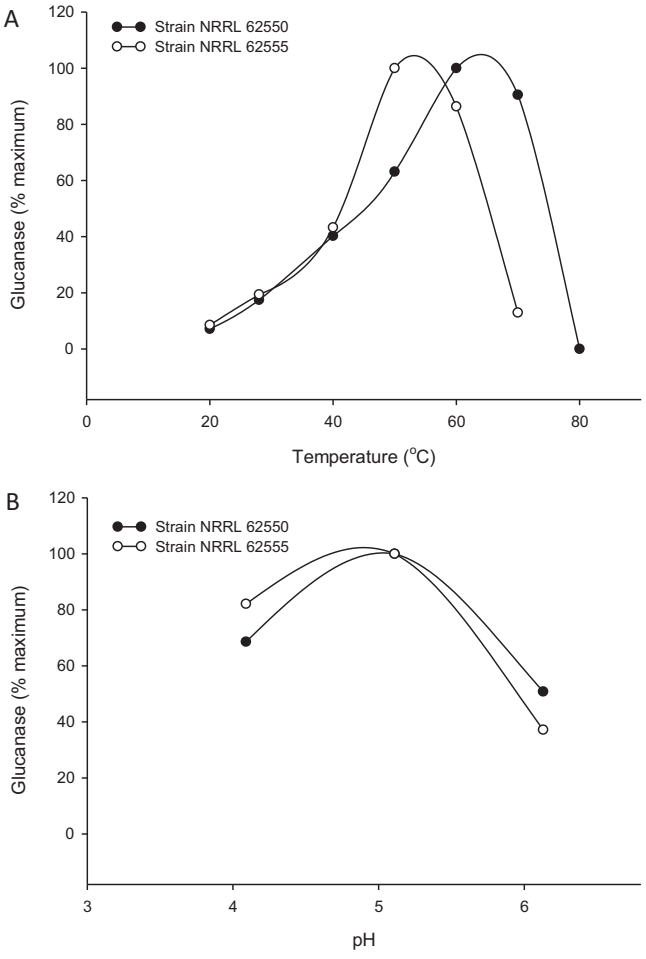

Fig. 1. Temperature (A) and pH (B) optima of β-glucanases from *Penicillium simplicissimum* strain NRRL 62550 and *Hypocrea nigricans* strain NRRL 62555.

3.2. Identification of novel strains producing β-glucanase activity against schizophyllan

Ten novel isolates producing β-glucanase activity against schizophyllan were identified by sequence analysis and colonial

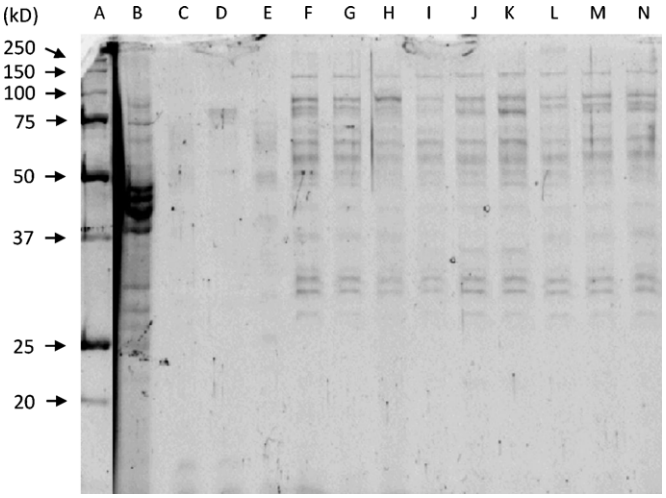

Fig. 2. SDS PAGE of cell-free culture supernatants. (A) BioRad Precision Plus protein standards; (B) commercial laminarinase from *Trichoderma* sp. (dil 10× from 12 U/mL); (C–E) replicate cultures of *Schizophyllan commune* ATCC 38548; (F–H) replicate cultures of *Penicillium crustosum* strain NRRL 62557; (I–K) replicate cultures of *P. crustosum* strain NRRL 62558; (L–N) replicate cultures of *P. crustosum* strain NRRL 62559.

Table 3
Specificity of β-glucanase activities produced by novel isolates grown on schizophyllan as a sole carbon source.

	β-glucanase activity (IU/mL) against substrates:					
Strain number	Schizophyllan[a]	Laminarin	Paramylon	Curdlan	Barley β-glucan	Protein (mg/mL)
1-1	0.24 ± 0.02	0.60 ± 0.12	0.011 ± <0.001	0.23 ± 0.01	0.98 ± 0.13	0.089 ± 0.01
1-2	0.16 ± 0.03	0.34 ± 0.12	0.004 ± <0.001	0.34 ± 0.04	0.60 ± 0.05	0.082 ± 0.01
1-3	0.17 ± 0.03	0.63 ± 0.04	0.005 ± <0.001	0.24 ± 0.02	0.68 ± 0.02	0.087 ± 0.00
6-1	0.008 ± <0.001	0.10 ± 0.03	0.002 ± <0.001	0.011 ± <0.001	0.036 ± <0.001	0.094 ± 0.00
6-2	0.002 ± <0.001	0.002 ± <0.001	0.001 ± <0.001	0.002 ± <0.001	0.02 ± 0.01	<0.01
6-3	0.21 ± 0.05	0.57 ± 0.04	0.005 ± <0.001	0.40 ± 0.02	0.73 ± 0.13	0.097 ± 0.01
6-4	0.19 ± 0.01	0.47 ± 0.07	0.005 ± <0.001	0.34 ± 0.02	0.62 ± 0.01	0.074 ± 0.01
9-1	0.026 ± <0.001	0.24 ± 0.03	0.013 ± <0.001	0.029 ± <0.001	0.32 ± 0.05	0.078 ± 0.02
9-2	0.032 ± <0.001	0.16 ± 0.07	0.004 ± <0.001	0.037 ± 0.01	0.34 ± 0.03	0.081 ± 0.01
9-3	0.029 ± <0.001	0.19 ± 0.01	0.010 ± <0.001	0.034 ± <0.001	0.24 ± 0.03	0.052 ± 0.01
ATCC 38548	0.003 ± <0.001	0.10 ± 0.04	<0.001	0.013 ± <0.001	0.26 ± 0.01	0.050 ± 0.04

[a] Activities against schizophyllan from Table 1.

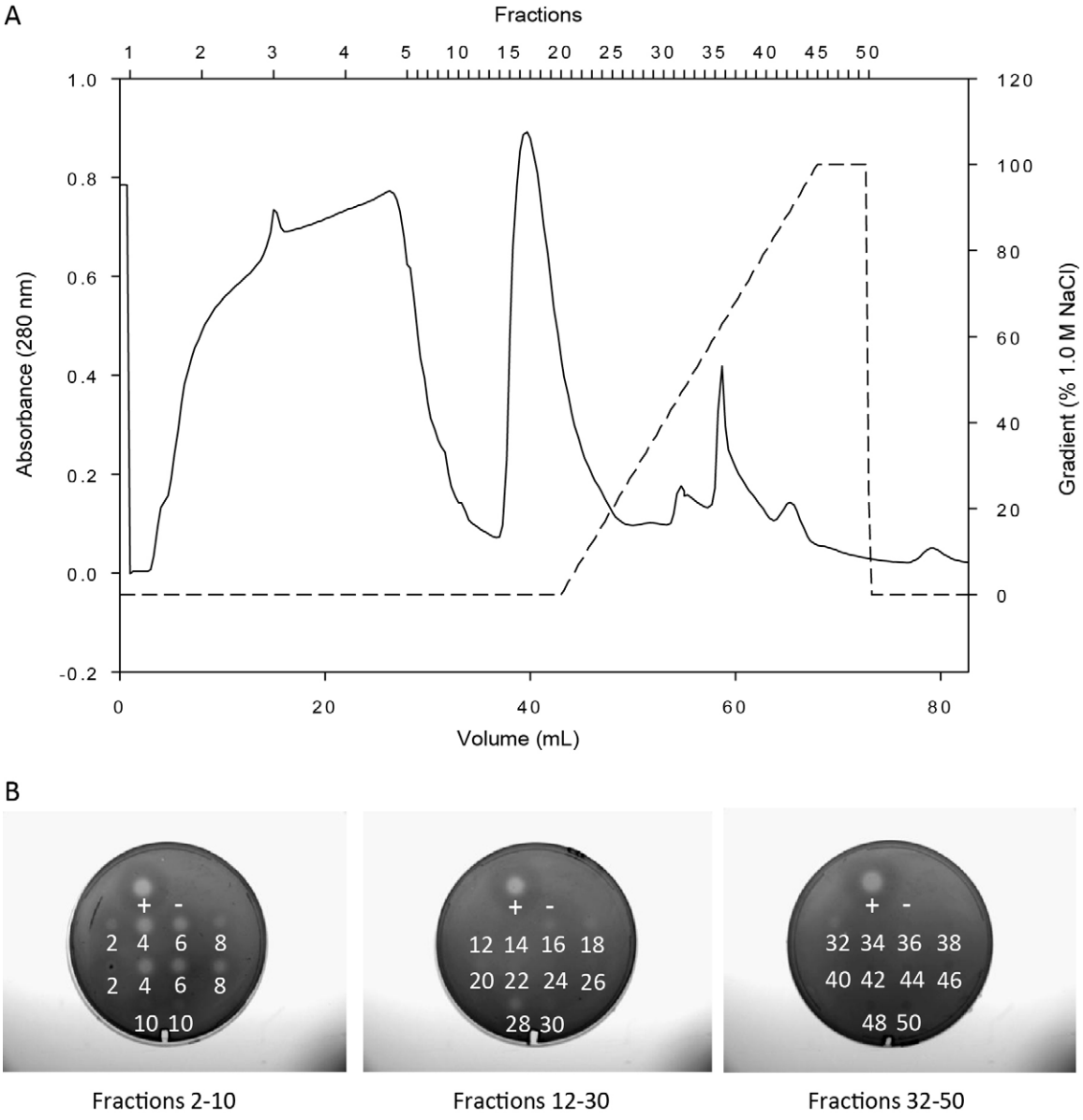

Fig. 3. Partial purification of β-glucanase from *Penicillium crustosum* strain NRRL 62558. (A) High Q anion-exchange column; (B) Rapid, semi-quantitative β-glucanase assays of High Q fractions (+ = laminarinase control, – = water control).

Table 4
Activity of commercial β-glucanases against schizophyllan and other β-glucans.

β-glucanase activity (IU/mL) against substrates						
Enzyme	Schizophyllan	Laminarin	Paramylon	Curdlan	Barley β-glucan	Protein (mg/mL)
Cellulase, *Aspergillus niger*	<0.001	<0.001	0.47 ± 0.01	0.50 ± 0.06	5.4 ± 0.01	0.05 ± <0.001
Cellulase, *Trichoderma viride*	<0.001	<0.001	0.07 ± <0.001	0.24 ± 0.08	3.8 ± 0.01	0.18 ± <0.001
β-glucanase, *A. niger*	<0.001	<0.001	0.09 ± 0.01	0.35 ± 0.01	30 ± <0.001	3.5 ± 0.02
β-glucanase, *T.longibrachiatum*	0.15 ± 0.01	<0.001	0.05 ± <0.001	0.45 ± 0.03	63 ± 0.03	1.9 ± <0.001
β-glucanase, *Bacillus subtilis*	<0.001	<0.001	<0.001	<0.001	58 ± 0.01	1.2 ± <0.001
Laminarinase, *Trichoderma* sp.	4.7 ± 0.02	46 ± 0.01	0.70 ± 0.02	19 ± 0.02	200 ± 0.01	11 ± 0.01

morphologies (Table 2). One isolate from a woodland isolation site was identified as a *Penicillium simplicissimum*, while two were shown to be *Hypocrea nigricans*. Two isolates from clay soil were identified as *Aspergillus flavus* and *A. alliaceus*, while the rest were *H. nigricans.* All three strains isolated as laboratory contaminants of stored schizophyllan solutions were identified as *Penicillium crustosum* (Table 2). Thus, most novel isolates producing β-glucanase activity against schizophyllan appeared to belong to either *Penicillium* species or *H. nigricans*. These genera are well known for production of numerous hydrolytic enzymes, including β-glucanases known to be active against β-glucans other than schizophyllan.

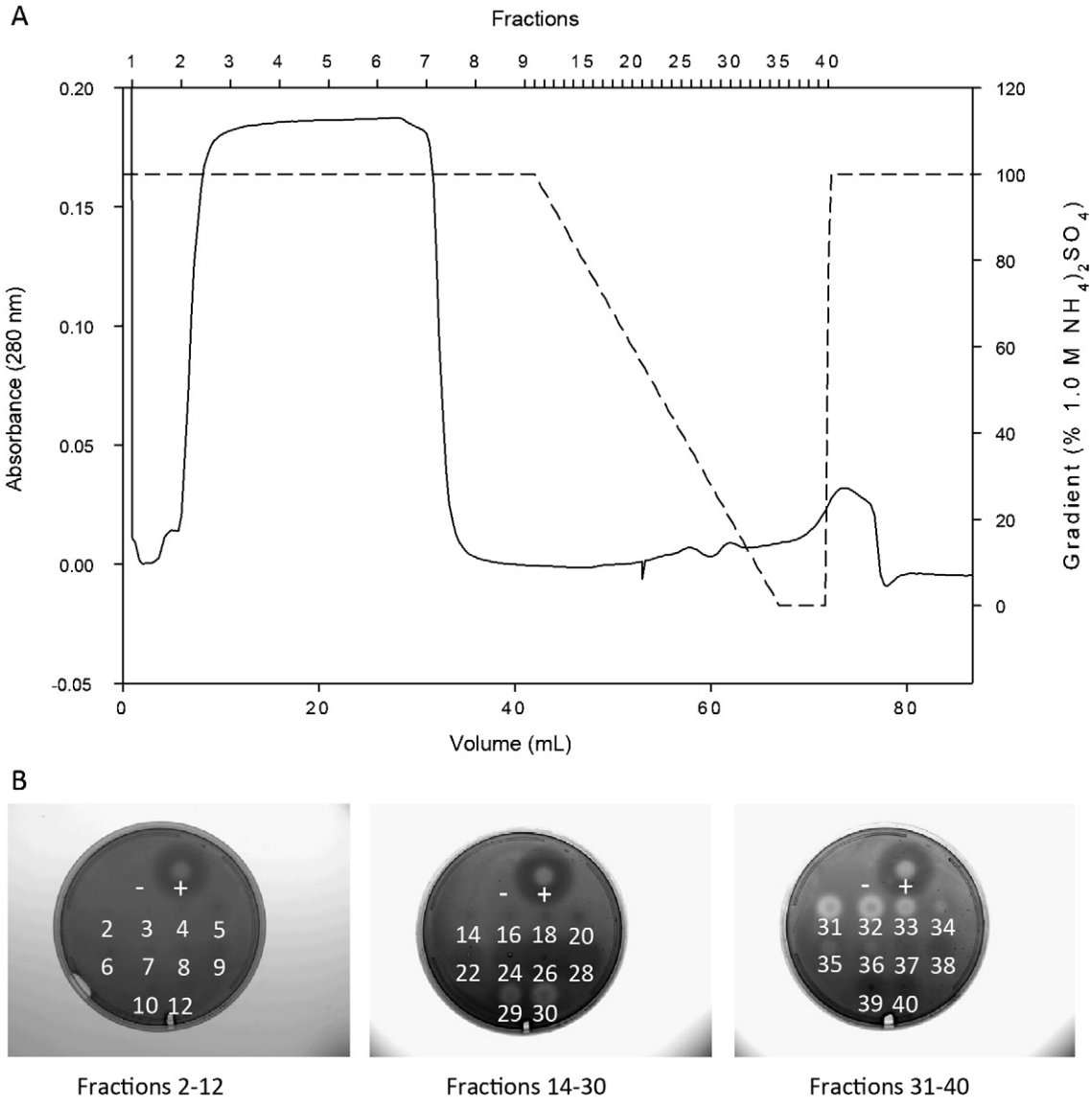

Fig. 4. Partial purification of β-glucanase from *Penicillium crustosum* strain NRRL 62558. (A) Phenyl Sepharose column; (B) Rapid, semi-quantitative β-glucanase assays of phenyl sepharose fractions (+ = laminarinase control, − = water control).

3.3. Specificity of β-glucanase activities produced by novel isolates

The specificity of β-glucanase activities produced by novel isolates grown on schizophyllan as a sole carbon source was tested in assays on other β-glucans, specifically curdlan, laminarin, paramylon, and barley β-glucan (Table 3). Enzymes were also tested from *S. commune* strain ATCC 38548 cultured on schizophyllan. *S. commune* has been reported to produce cellulase [17] and β-glucanase with activity against schizophyllan [5], and it is thought that the organism can consume the polysaccharide as a carbon source in late cultures [2]. While schizophyllan possesses a β-(1,3) backbone with regular β-(1,6) branches of one subunit [2], curdlan is a water-insoluble β-glucan produced by *Agrobacterium* sp. (*Alcaligenes faecalis*), composed primarily of linear β-(1,3) linkages [18]. Laminarin, from the brown alga *Laminaria digitata*, is a water soluble, mainly linear β-(1,3) glucan with a small number of β-(1,6) linked side chains [19]. Paramylon is similar to laminarin, but has a much higher level of crystallinity [20]. Barley β-glucan is a linear homoglucan, mostly composed of two or three consecutive β-(1,4) linkages separated by a single β-(1,3) linkage [21].

β-glucanase activities from novel isolates were generally 2–10 times higher on laminarin than on schizophyllan (Table 3). Since laminarin is a water-soluble β-(1,3)-glucan with far fewer β-(1,6) side chains than schizophyllan, this result suggests that the novel isolates produce primarily β-(1,3)-endoglucanases that attack the backbone of schizophyllan, and that the abundant side chains of schizophyllan limit access to this backbone. However, activities against paramylon were uniformly lower than on schizophyllan. Although the structure of paramylon is similar to that of laminarin, its higher degree of crystallinity may also limit access to enzymes. Activities against curdlan were equivalent to double those on schizophyllan, depending on the strain used. Curdlan completely lacks side chains, and the polymer is consequently insoluble. This may explain activities intermediate between those of schizophyllan and laminarin. Interestingly, enzymes from novel isolates were generally more active against barley β-glucan than against laminarin (Table 3). Since barley β-glucan does not contain consecutive β-(1,3) linkages, this could mean that either β-(1,3)-endoglucanases are able to recognize single linkages, or that novel isolates also produce β-(1,4)-endoglucanases that attack the β-(1,4) linkages in barley β-glucan. Under conditions tested here, *S. commune* produced only marginal activity against schizophyllan.

3.4. Activity of commercial β-glucanases against schizophyllan and other β-glucans

To further test the action of endoglucanases against schizophyllan, a set of commercial enzymes was tested against the same substrates. Cellulases from *A. niger* and *T. viride* hydrolyze the β-(1,4) linkages in cellulose (Calbiochem). β-glucanases from *A. niger* and *B. subtilis* degrade β-1,4-glucans of cellulose and possibly other polymers (Sigma–Aldrich). β-glucanase from *T. longibrachiatum* is a mixture of enzymes composed mainly of β-(1,3)/β-(1,4) glucanase, xylanase, and cellulase (Sigma–Aldrich). Laminarinase from *Trichoderma* sp. is defined by its activity on laminarin. However, it is possible that all of these commercial preparations include multiple enzyme activities. For comparison purposes, all commercial enzymes were prepared at or diluted to12 U activity/mL, based on advertised activities, in 50 mM sodium acetate buffer, pH 5.0. Assays were then run on schizophyllan and other β-glucans.

All commercial enzymes tested were most active against barley β-glucan (Table 4). Cellulases also exhibited activity against curdlan and paramylon. Interestingly, cellulase from *A. niger* was equally active against these substrates, despite the higher crystallinity of paramylon. Fungal β-glucanases also showed activity against curdlan and paramylon, although β-glucanase from *B. subtilis* did not. Results suggest that different commercial enzyme preparations vary in enzyme composition and specificity.

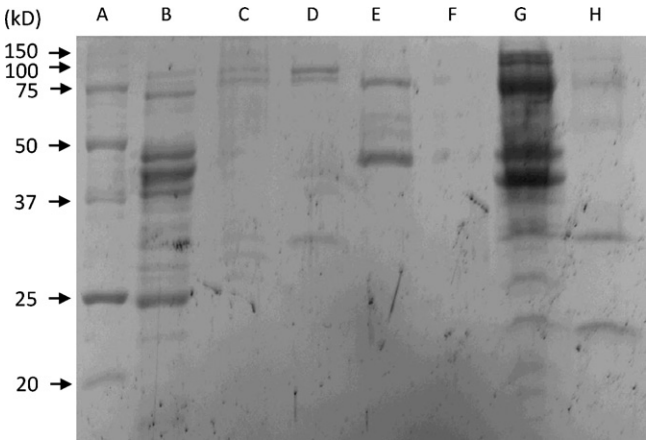

Fig. 5. SDS PAGE gel of partially purified β-glucanase fractions. (A) BioRad Precision Plus protein standards; (B) commercial laminarinase from *Trichoderma* sp. (dil 10× from 12 U/mL); (C) *Penicillium crustosum* strain NRRL 62558 total cell-free culture supernatant; (D) *P. crustosum* strain NRRL 62558 Phenyl Sepharose column Area 1 (fraction 32); (E) *P. crustosum* strain NRRL 62558 High Q column Area 3 (fraction 32); (F) *P. crustosum* strain NRRL 62558 High Q column Area 2 (combined fractions 16–24); (G) *P. simplicissimum* strain NRRL 62550 total cell-free culture supernatant; (H) *P. simplicissimum* strain NRRL 62550 High Q column fraction 34.

Among commercial enzyme preparations tested, only β-glucanase from *T. longibrachiatum* and laminarinase from *Trichoderma* sp. exhibited activity against schizophyllan (Table 4). These enzymes were 3–4 times more active against curdlan than against schizophyllan (Table 4). Curdlan may be a more accessible substrate than schizophyllan, due to its lack of side chains. However, only laminarinase from *Trichoderma* sp. also exhibited activity against laminarin. Specific activities against schizophyllan were 0.1 U and 0.4 U/mg protein for β-glucanase from *T. longibrachiatum* and laminarinase from *Trichoderma* sp., respectively. These activities are similar to those from novel *P. crustosum* strains NRRL 62557, NRRL 62558, and NRRL 62559, but lower than specific activities from novel *P. simplicissimum* strain NRRL 62550 and novel *H. nigricans* strains NRRL 62551, NRRL 62552, NRRL 62555, and NRRL 62556, which ranged from 2.0 to 2.7 U/mg protein.

3.5. pH and temperature optima of β-glucanase produced by novel isolates

pH and temperature optima were determined for β-glucanases from *P. simplicissimum* strain NRRL 62550 and *H. nigricans* strain NRRL 62555. Cell-free culture supernatants from strains NRRL 62550 and NRRL 62555 exhibited rather sharp temperature optima at about 60 °C and 50 °C, respectively (Fig. 1A). Both strains showed a broad pH optimum centered at about pH 5.0 (Fig. 1B). Thus, β-glucanases from novel isolates appear to be relatively thermophilic.

3.6. Partial purification of β-glucanase from P. crustosum

β-glucanase activities were partially purified from *P. crustosum* strain NRRL 62558. Cell-free culture supernatants from *P. crustosum* strains NRRL 62557, NRRL 62558, and NRRL 62559 showed similar protein patterns on SDS-PAGE, and quite different from those from *S. commune* and commercial laminarinase (Fig. 2). All of these samples produced clear zones when spotted on schizophyllan substrate plates, indicating that they contained endo-glucanase

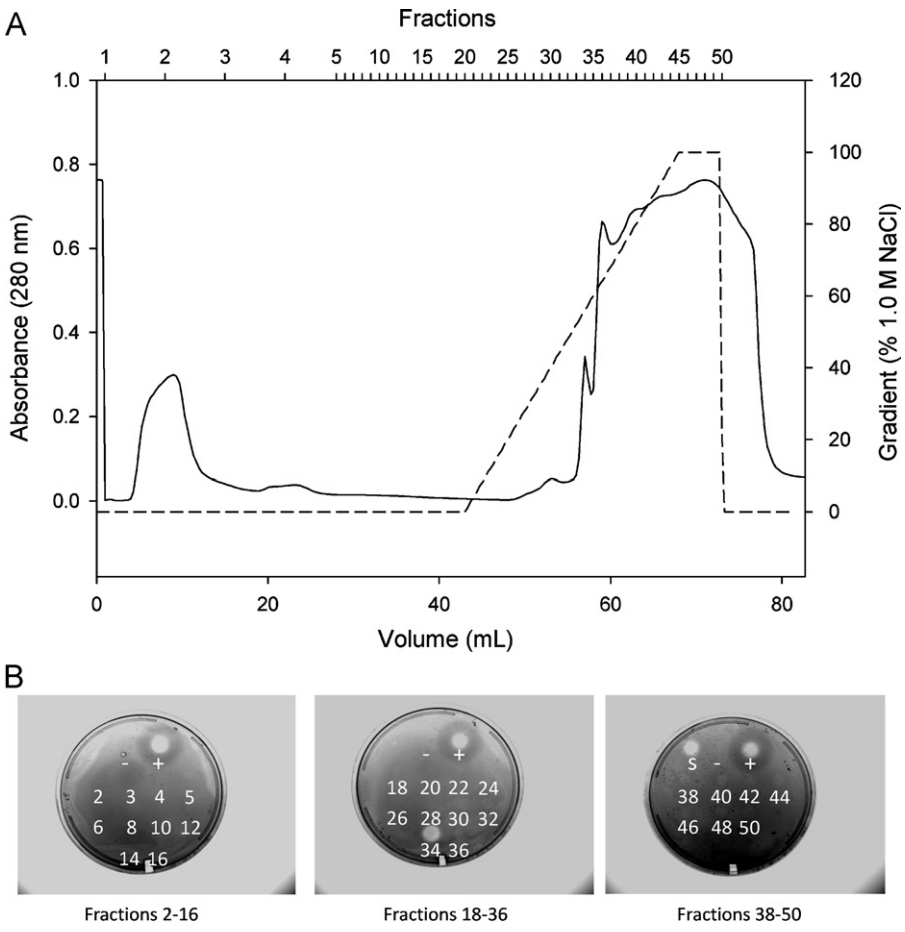

Fig. 6. Partial purification of β-glucanase from *P. simplicissimum* strain NRRL 62550. (A) High Q anion-exchange column; (B) Rapid, semi-quantitative β-glucanase assays of High Q fractions. (+ = laminarinase control, − = water control, S = culture supernatant control).

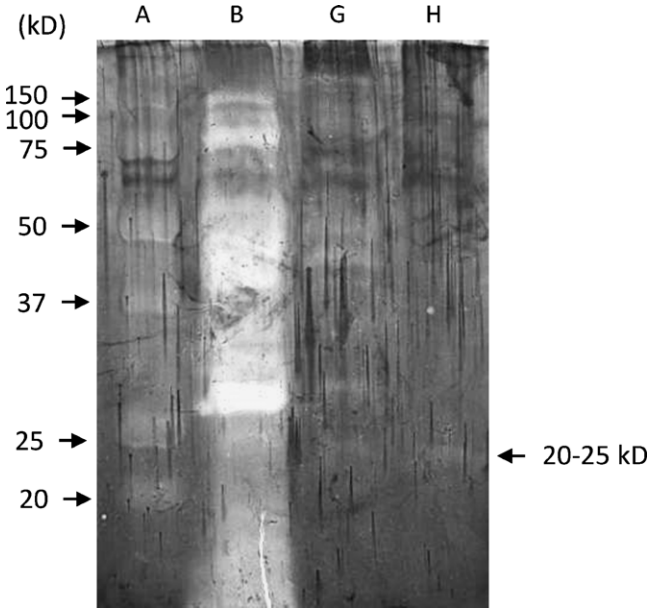

Fig. 7. Zymogram of β-glucanase activities in SDS PAGE gel of partially purified β-glucanase from *P. simplicissimum* strain NRRL 62550. (A) BioRad Precision Plus protein standards; (B) commercial laminarinase from *Trichoderma* sp. (dil 10× from 12 U/mL); (G) *Penicillium simplicissimum* strain NRRL 62550 total cell-free culture supernatant; (H) *P. simplicissimum* strain NRRL 62550 High Q column fraction 34.

activities against this substrate (data not shown). Culture supernatant from strain NRRL 62558 was subjected to chromatography using a High Q anion exchange column (Fig. 3A). β-glucanase activity was measured by spotting column fractions on a schizophyllan substrate plate (Fig. 3B). Three active fractions were identified: High Q Area 1 included fractions 2–10, representing a broad protein peak; High Q Area 2 included fractions 16–22, representing a large distinct protein peak; and High Q Area 3 included fractions 28–32, representing a small but distinct protein peak. High Q Area 1 was further purified on a Phenyl Sepharose column (Fig. 4). β-glucanase activity was associated with fraction 29–36, represented by a small distinct protein peak (Phenyl Sepharose Area 1). Active fractions from each of the partially purified peaks were combined, desalted, and subjected to SDS-PAGE (Fig. 5). Phenyl Sepharose Area 1 (lane D) was enriched in protein species of approximately 30 kD and 100 kD, also apparent in unfractionated culture supernatants (lane C). On the other hand, High Q Areas 2 and 3 (lanes F and E, respectively) were enriched in protein species of about 40 kD and 75 kD, not sufficiently abundant to be evident in culture supernatants. Thus, β-glucanase activity in *P. crustosum* appears to be associated with multiple protein species. Although little is known about enzymes that specifically attack schizophyllan, a survey of 160 fungi found that fungal laminarinases often have multiple components [22].

3.7. Partial purification of β-glucanase from P. simplicissimum

Cell free culture supernatant from *P. simplicissimum* strain NRRL 62550 was subjected to chromatography using a High Q anion

exchange column, and β-glucanase activity was measured by spotting column fractions on a schizophyllan substrate plate (Fig. 6). Unlike *P. crustosum* strain NRRL 62558, which showed multiple active fractions associated with multiple protein species, strain NRRL 62550 exhibited a single active High Q fraction (34), associated with a single protein peak. This fraction was desalted and concentrated ten-fold, and subjected to SDS-PAGE (Fig. 5, lane H). Fraction 34 included protein species at approximately 20–25 kD; 25–37 kD; 50–75 kD; 75 kD; and 100 kD. These proteins were also apparent in unfractionated cell-free culture supernatant (Fig. 5, lane G), indicating that they are abundant species.

Proteins from *P. simplicissimum* strain NRRL 62550 were also subjected to zymogram analysis, using a duplicate gel that incorporated schizophyllan into its matrix (Fig. 7). Zymogram gels clearly showed many clear zone bands both in the positive control (laminarinase from *Trichoderma* sp., lane B) and the unfractionated culture supernatant (lane G). Partially purified fraction 34 (lane H) showed only a single prominent band of active protein of 20–25 kD (lane H). Since only fraction 34 showed β-glucanase activity on a schizophyllan substrate plate, this 20–25 kD species may be relatively more active than other β-glucanases from this strain.

4. Conclusions

Although schizophyllan is a well-known and commercially valuable polysaccharide, little is known about β-glucanases that can attack this structure. Such enzymes could be valuable to modify schizophyllan for novel applications. In this study, novel fungi were isolated that grew on schizophyllan as a sole carbon source. Several produced β-glucanase activities against schizophyllan, particularly isolates of *P. simplicissimum*, *P. crustosum*, and *H. nigricans*. Partial purifications demonstrated the presence of multiple active endoglucanase species. Results indicate that novel fungi are a promising source for schizophyllan-degrading enzymes.

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